Defense-in-depth by mucosally administered anti-HIV dimeric IgA2 and systemic IgG1 mAbs: complete protection of rhesus monkeys from mucosal SHIV challenge

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Abstract

Background: Although IgA is the most abundantly produced immunoglobulin, its role in preventing HIV-1 acquisition, which occurs mostly via mucosal routes, remains unclear. Data from the RV144 trial implied that vaccine-induced plasma IgA antibodies (Abs) counteracted the protective effector mechanisms of IgG1 Abs with the same epitope specificity; mucosal samples were not available for study. We previously performed passive mucosal immunizations in rhesus macaques (RMs) with the HIV-1 envelope (Env)-specific, neutralizing monoclonal antibody (mAb) HGN194. The dimeric IgA2 (dIgA2) form of the mAb administered intrarectally (i.r.) protected only 17% of the RMs, whereas the dimeric IgA1 (dIgA1) version also given i.r. prevented infection in 83% of the macaques after i.r. challenge with simian-human immunodeficiency virus (SHIV). We hypothesized that mucosal dIgA2 might diminish the protection provided by IgG1 mAbs targeting the same epitope.

Results: To test our hypothesis, we compared intravenously (i.v.) administered HGN194 IgG1 either alone or in combination with the dIgA2 version given i.r.. Both mAb forms used as single agents and the combination of the two neutralized the challenge virus equally well in vitro. None of the RMs given i.v. HGN194 IgG1 alone remained virus-free. In contrast, all RMs given the HGN194 IgG1+dIgA2 combination were completely protected against high-dose i.r. SHIV-1157ipEL-p challenge.

Conclusion: Combining suboptimal defenses at the mucosal and systemic levels can completely prevent virus acquisition in all animals. These data imply that active vaccination should focus on defense-in-depth, a strategy that seeks to build up fortified defensive fall-back positions well behind the armed frontline.

Keywords: IgA, dimeric IgA2, IgG, complete protection, macaque model, passive immunization, non-human primate model, mucosal challenge, SHIV, HIV

Background

The partially successful RV144 trial has opened new horizons for HIV-1 vaccine design while posing new challenges for researchers [1]. Post-trial analyses revealed an inverse correlation between IgG antibodies (Abs) specific for the variable loops 1 and 2 (V1V2) of the HIV-1 envelope (Env) and the risk of HIV-1 infection. A direct correlation between plasma Env-specific IgA and the risk of HIV-1 infection was also observed [2], although vaccinees with high Env-specific plasma IgA were not more likely to become infected than placebo recipients. Additionally, antibody-dependent cellular cytotoxicity (ADCC) responses in the presence of low plasma concentration of anti-Env IgA correlated with reduced risk of infection. These findings suggested that Env-specific circulating IgA impeded the protective effects of IgG Abs. Secondary analyses showed that vaccinees with plasma IgA specific to the first conserved region (C1) of HIV-1 Env gp120 had a higher risk of infection than vaccinees without C1-specific IgAs [2]. C1-specific monoclonal Abs (mAbs) isolated from RV144 vaccinees and expressed as IgG1 showed HIV-1-specific ADCC-mediated cell killing [3]. Of note, two of these ADCC-mediating IgG1 mAbs, namely CH29 and CH38, were originally of IgA2 and IgA1 isotypes, respectively. Later, Tomaras et al. demonstrated that the C1 epitope recognized by total plasma IgA and mAbs CH29 and CH38 expressed as IgA2 overlapped with the epitopes of IgG1 mAbs within the same Env region [4]. Remarkably, mAb CH38 expressed as IgA2 (originally IgA1) inhibited ADCC activity of C1-specific IgG1 mAbs isolated from RV144 vaccinees, while mAb CH29 expressed as IgA2 (originally IgA2) did not [4]. Since mucosal samples had not been collected during

84 the RV144 trial, the question remains as to how IgA2 and IgG1 with the same epitope
85 specificity would interact in the mucosal compartment.

86 Most existing vaccines are administered intramuscularly or subcutaneously and
87 induce both systemic IgG and IgA antibody responses. However, robust mucosal IgA
88 responses with such vaccines are rarely generated [reviewed in [5]]. In contrast,
89 intranasal and oral vaccination strategies induce strong mucosal IgA as well as serum
90 IgG responses and have been successfully implemented against the number of
91 infectious agents [reviewed in [5, 6]]. Mucosal immunization of rhesus monkeys (RMs)
92 with HIV or SIV antigens led to the development of specific IgA responses in vaginal
93 and rectal fluids [7-9]. RMs immunized via both the intramuscular and intranasal routes
94 with HIV-1 gp41-subunit antigens grafted on virosomes were completely protected from
95 persistent systemic infection with SHIV-SF162P3 and showed gp41-specific vaginal
96 transcytosis-blocking IgAs as well as vaginal IgGs with neutralizing and/or antibody-
97 dependent cellular-cytotoxicity activities [10].

98 IgA is the most abundant immunoglobulin (Ig) class: each day, the human body
99 generates more IgA than all other Ig classes combined [reviewed in [11]]. IgA is mostly
100 produced in lymphoid tissues associated with mucosal surfaces; IgA production in the
101 gut amounts to 40–60 mg kg^{-1} day^{-1} [reviewed in [12]]. Humans have two IgA isotypes –
102 IgA1 and IgA2 – and both are presented as dimers or polymers at mucosal sites. HIV-1
103 Env-specific IgA1 has been found in cervicovaginal secretions of highly exposed
104 persistently seronegative (HEPS) women [13, 14]. Moreover, HEPS men who had sex
105 with men developed serum IgA1 recognizing HIV-1 Env after oral exposure to HIV-1
106 [15] and HIV-1-specific IgA was found in cervicovaginal fluids of repeatedly exposed sex

workers [16, 17]. Furthermore, mucosal IgA Abs specific to HIV-1 gp41 were associated with protection against HIV-1 infection in seronegative partners of HIV-1-infected individuals [18].

The RM/primate immunodeficiency virus model is widely used for HIV-1 vaccine research to reflect vaccination of HIV-1-naïve individuals. However, it is likely that most people are exposed to live HIV-1 without becoming systemically infected. Thus, the ability of the naïve RM model to predict possible outcomes in live-HIV-experienced humans is unknown. In this regard, a non-human primate model using simian-human immunodeficiency virus (SHIV)-exposed but uninfected macaques will reflect the real-life situation where prospective recipients of an AIDS vaccine are not naïve but have a history of HIV-1 exposure that did not result in seroconversion.

In our recent RM study, passive intrarectal (i.r.) immunization with a dimeric IgA1 (dIgA1) version of the anti-V3 loop crown mAb, HGN194 [19], completely protected five out of six RMs against i.r. SHIV challenge [20]. In contrast, the dIgA2 form of the same mAb protected only one out of six RMs. In the same study, the IgG1 version of HGN194 given i.r. prevented infection in two out of six passively immunized animals. The proposed mechanism of differential protection was better virion capture by dIgA1 compared with dIgA2 due to the longer hinge region of dIgA1.

It is worth mentioning that the ratio of IgA1 and IgA2 varies in different human mucosal fluids, with IgA1 percentages in male genital secretions and nasal fluids reaching 80-90% and 60% in saliva. Female genital secretions and rectal fluids contain approximately 60% IgA2 [reviewed in [21]]. Among primates, only some great apes have two IgA isotypes, and all macaques, including RMs, possess only one IgA version,

which is structurally similar to human IgA2 [22]. In this regard, assessing the relationship between mucosal IgA2 and systemic IgG with the same epitope specificity and elucidating the role of this combination against immunodeficiency virus acquisition is important and timely.

The potentially negative role of IgA responses in the RV144 trial mentioned above led us to hypothesize that mucosal dimeric IgA2 could compromise the protective effect of IgG1 of the same epitope specificity. Here, we present the results of a study using systemic infusion of IgG1 and i.r. application of dIgA2 mAbs with the same epitope specificity performed in RMs that had been previously exposed to SHIV but remained aviremic and seronegative. Unexpectedly, all RMs treated with the combination of HGN194 IgG1+dIgA2 were completely protected against mucosal SHIV challenge.

Results

Animal selection and analysis of immune responses

The current study used RMs that had remained aviremic and seronegative during two separate, earlier experiments involving passive immunization with mAb HGN194 followed by i.r. SHIV challenge. The human IgG1 neutralizing mAb (nmAb) HGN194, isolated from a long-term non-progressor infected with HIV-1 clade AG, targets the V3-loop crown and protects against cross-clade SHIV challenge *in vivo* [19, 23]. The use of previously exposed animals recapitulates the common scenario in humans, where any given HIV-1 exposure results in a low incidence of systemic infection and where non-

152 transmitting exposures result in local and systemic immune responses in some
153 individuals.

154 The first study involved topical (i.r.) application of HGN194 dIgA1, dIgA2 or IgG1
155 [20]. A second, unpublished experiment sought to elucidate the role of nmAb effector
156 functions in protection agaisnt i.r. SHIV challenge. In this second study, RMs had been
157 treated intravenously (i.v.) with wild-type HGN194 IgG1 ($IgG1_{wt}$), its LALA mutant
158 ($IgG1_{LALA}$) in which binding to the Fcγ receptor (FcγR) was abrogated thereby deleting
159 effector functions, or with an afucosylated version ($IgG1_{kif}$) of HGN194 IgG1 that had
160 increased binding to FcγRIII, respectively (unpublished data). In both studies, the
161 macaques had been challenged i.r. with 31.5 50% animal infectious doses (AID_{50}) of the
162 R5 clade C SHIV-1157ipEL-p [24].

163 All RMs selected for the current study were persistently aviremic (as measured
164 by a sensitive RT-PCR assay [25]) and were seronegative by SIV Gag ELISA (data not
165 shown). We assessed preselected animals for cellular and humoral immune responses
166 to viral proteins. SIV Gag-specific proliferative $CD4^+$ and $CD8^+$ T-cell responses were
167 measured at four to eight weeks after the earlier viral challenge (Figure 1). Although
168 persistently aviremic, 9 out of 14 macaques had measurable proliferation of $CD4^+$ cells
169 and 11 animals showed $CD8^+$ proliferation in responses to stimulation with SIV Gag
170 peptides; three RMs had marginal responses and animal RBk-14 showed no reaction.
171 In general, proliferation of $CD8^+$ cells was weaker than $CD4^+$ lymphocytes.

172 To analyze possible humoral immune responses among the preselected RMs,
173 we first analyzed the residual plasma concentration of HGN194 IgG1 in all animals that
174 had received the HGN194 mAbs systemically in a previous, unpublished study (Figure

2A). At week 8 after the mAb passive transfer and virus challenge, the concentration of HGN194 $IgG1_{wt}$ was about 2 µg/ml and the $IgG1_{kif}$ concentration was marginally above background. Four weeks later, $IgG1_{wt}$ was detected as low as at 0.14, 0.07 and 0.08 µg/ml in the plasma of RMs RBk-14, REo-14, and RIk-14, respectively. At the same time, the mAb concentration in the plasma of HGN194 $IgG1_{kif}$ -treated macaques fell below the detection limit. Of note, the average *in vivo* 90% plasma inhibitory concentration (IC_{90}) of HGN194 IgG1 was estimated at 2.15 µg/ml [23]. To allow complete clearance of previously infused mAbs, the current experiment was scheduled at 16 - 18 weeks after the initial mAb administration. Additionally, we tested plasma samples of animals RBk-14, REo-14 and RIk-14 collected on the day of new mAb administration for any remaining HGN194 IgG1. As expected, no mAb was detected just before re-administration (not shown).

Next, we analyzed the RMs that had received the human mAbs HGN194 $IgG1_{wt}$ and $IgG1_{kif}$ systemically for possible anti-human IgG antibody responses. During recurrent administrations of human IgG, these anti-species Abs, if developed, might cause adverse reactions and rapid elimination of human mAbs from the circulation. Importantly, none of the HGN194-treated RMs had developed any RM anti-human IgG Ab responses (Figure 2B) as a consequence of their prior treatment with HGN194 IgG1.

Using ELISA, we next confirmed that the passively immunized, protected RMs had not mounted any anti-HIV-1 Env Ab responses of their own (Figures 2C and D). The secondary Ab in the ELISA was specific for RM IgGs only. No reactivity was seen, as expected from the negative SIV Gag ELISA data (not shown). Thus, the animals did

not have any Ab responses that may have skewed the new passive immunization/SHIV challenge study.

Group assignment and study design

Animals that had earlier received different versions of HGN194 mAb through different routes were distributed evenly between two new experimental groups (Table 1). Each group contained two RMs that had received HGN194 IgG1 systemically and four RMs treated topically. The control group consisted of two macaques that had received HGN194 IgG1 systemically. MHC alleles and TRIM5α genotypes also were distributed evenly among the new groups (Table 1).

The experimental timeline of the current study is depicted in Figure 3. RMs in both Groups A and B received HGN194 IgG1 i.v. at 1.45 mg/kg 24 h before the viral challenge. RMs of Group A were additionally treated with 1.25 mg of HGN194 dIgA2 applied i.r. (1.25 mg of mAb in 2.1 ml of phosphate-buffered saline (PBS)) 30 min before the virus challenge. Control Group C macaques were left untreated. All animals were challenged i.r. with 31.5 AID_{50} of SHIV-1157ipEL-p [24], an R5 clade C SHIV, and monitored prospectively by measuring of plasma viral RNA (vRNA) loads.

The combination of IgG1+dIgA2 versions of HGN194 completely protects RMs from single high-dose SHIV challenge

The single mucosal high-dose challenge with SHIV-1157ipEL-p resulted in systemic infection of all macaques of Group B (HGN194 IgG1 only) by week 3 (Figure 4A). Control animals (Group C) were viremic as well. Surprisingly, all Group A RMs, which

had received the combination of IgG1+dIgA2, remained aviremic. The time to vRNA load >50 copies/ml for Groups A and B animals was compared by Kaplan-Meier analysis using the log-rank test with two-sided P-values (Figure 4B). The combination of IgG1+dIgA2 demonstrated significantly better protection against mucosal SHIV-1157ipEL-p challenge compared with IgG1 alone (P = 0.0005). The shorter half-life of IgG1 in Group B RMs can be explained by absorption and removal of IgG1 from the circulation by newly replicating virus.

In our previous experiment, the same virus challenge caused systemic infection of five out six RM treated with the same dose of dIgA2 i.r. [20]. The results of another, yet unpublished study with different IgG1 versions of HGN194 demonstrated infection of four out seven macaques infused i.v. with 1 mg/kg of $IgG1_{wt}$. Taken together, these results indicate that the combination of systemic IgG1 and topical dIgA2 treatments yielded better protection compared with individual mAb treatment alone.

HGN194 IgG1 pharmacokinetics and plasma neutralization capacity

The IgG1 pharmacokinetics were analyzed by ELISA. The infused IgG1 mAb showed the classical circulation profile in both groups of macaques (Figure 5A) following virus challenge. RMs of Group B cleared IgG1 faster compared with animals of Group A (Figure 5B). Mean half-lives of HGN194 IgG1 were calculated at 15.8 ± 3.9 days for Group A and 8.2 ± 2.6 days for Group B RMs (P = 0.0087, Mann-Whitney test).

Although HGN194 IgG1 demonstrated shorter half-life in Group B RMs, mAb concentrations on the day 0, the day of virus challenge, were similar for Groups A and B animals (Table 2). These concentrations were comparable to that reported previously

243 [23] as well as to the IgG1 concentration observed during the previous experiment
244 (unpublished data). Mean HGN194 IgG1 concentrations were 3.3 ± 0.9 μg/ml for Group
245 A and 3.1 ± 0.5 μg/ml for Group B, respectively.
246 Plasma samples of Group A and B RMs collected on the day of virus challenge
247 were able to neutralize SHIV-1157ipEL-p, the challenge virus, with the same efficiency
248 as demonstrated by TZM-bl cell-based neutralization assays (Table 2). There was no
249 difference between mean 50% plasma inhibitory concentration (IC_{50}) values of Groups
250 A (0.5 ± 0.2 μg/ml) and B (0.4 ± 0.08 μg/ml) measured in RM plasmas on the day of
251 virus challenge. These results are in line with *in vivo* IC_{50} values observed previously
252 for HGN194 IgG administered to infant RMs at 1 mg/kg dose [23]. Also, these data
253 clearly demonstrate that, in spite of faster clearance of mAb by RMs of Group B, RMs of
254 Groups A and B maintained equal concentrations of IgG1 in plasma on the day of SHIV-
255 1157ipEL-p challenge and that mAb was able to neutralize the challenge virus with the
256 same efficiency. Of note, all RMs in Group B with the shorter half-life were viremic,
257 which probably resulted in faster clearance post-challenge due to immune complex
258 formation.
259
260 **Individual mAbs and combination of IgG1+dIgA2 show the same neutralization**
261 **profiles *in vitro***
262 To understand why the combination was more protective than treatment with individual
263 mAbs, we examined the neutralization of the challenge virus *in vitro* by the combination
264 of IgG1+dIgA2. Toward this end, we used TZM-bl, A3R5 and human peripheral blood
265 mononuclear cell (PBMC)-based assays (Figure 6A-C). For all three assays, the

differences between neutralization curves were not significant as evaluated by multiple *t*-tests and two-way ANOVA test for multiple comparisons (not shown).

It should be noted that in the neutralization assays, dIgA2 and IgG1 were used at the same mass concentrations. The concentration of the IgG+dIgA2 combination was the sum of mass concentrations of individual mAbs. Dimeric IgA2 has a molecular weight of ~315 kDa, compared to ~150 kDa for IgG1; thus, the molar concentration of dIgA2 taken at the same mass concentration as IgG1 is twofold lower than for IgG1. However, dIgA2 bears four Fab regions, and IgG1 has only two. Therefore, the dIgA2 solution with a twofold lower molar concentration than the IgG1 solution contained the same molar concentration of antigen combining sites as the IgG1 solution. These considerations explain the similar neutralization curves for HGN194 IgG1, dIgA2, and combination of both.

The combination of IgG1+dIgA2 does not inhibit virus transcytosis *in vitro*

As we previously reported [20], only HGN194 dIgA1, but not dIgA2 or IgG1 as single agent, was able to inhibit virus transcytosis in vitro. We evaluated whether the combination of HGN194 IgG1+dIgA2 could inhibit the transcytosis of SHIV-1157ipEL-p at pH 6. A low pH has been reported to enhance antibody-mediated virus transcytosis [26]. Additionally, the pH of colonic rectal fluid for *Macaca* species was reported to range between 5.1 and 7.8 [27, 28]. As shown, the mean concentration of dIgA2 administered i.r. was 231.6 μg/ml 30 min after topical application [20]. To reflect the *in vivo* observed concentration of dIgA2, we performed the transcytosis assay at 200 μg/ml of dIgA2 or dIgA1 used as a positive control mixed with different concentrations of

HGN194 IgG1 or the isotype control IgG1 Fm-6 (Figure 6D). At pH 6, HGN194 IgG1 alone or in combination with dIgA2 enhanced transcytosis of SHIV-1157ipEL-p across HEC-1A cells. As expected, dIgA1 used as a positive control completely reversed the effect of specific IgG1 and inhibited transcytosis almost completely at any IgG1 concentration tested. The fact that the IgG1+dIgA2 combination was unable to prevent enhanced virus transcytosis *in vitro* suggests that this combination is not likely to exert its protective effect through inhibition of transcytosis.

Discussion

We have shown that HIV-1 Env-specific IgG1 plasma Abs in combination with mucosal dIgA2 of the same epitope specificity completely protected RMs against high-dose mucosal challenge with SHIV-1157ipEL-p. Initially, we demonstrated that RMs that had remained aviremic in previous passive immunization experiments had developed low-level, virus-specific cell-mediated immune responses and thus represent a relevant model to assess immunization efficacy among HIV-1-exposed, uninfected individuals. The striking 100% protection we observed here in RMs with the combination of i.v. IgG1 plus i.r. dIgA2 mAbs was unexpected – given that the group treated with the IgG1 version alone had 0% protection and that i.r. dIgA2 had protected only 17% of RMs challenged with the same clade C SHIV earlier. Lastly, we demonstrated that inhibition of virus transcytosis, as suggested for dIgA1 [20], was unlikely to be the mechanism of protection by the combination of IgG1+dIgA2. Thus, our findings suggest that mucosal dimeric IgA2, if generated by active immunization, will complement HIV-1-specific

311 plasma IgG1 in preventing virus acquisition rather than diminishing the protective role of
312 plasma IgG1.

313 The gastrointestinal mucosa is the largest mucosal surface in the human body,
314 and it represents the major portal of HIV-1 entry during mother-to-child transmission via
315 breastfeeding, sexual transmission in men who have sex with men, as well as during
316 heterosexual anal intercourse [reviewed [29]]. The risk of HIV-1 transmission through
317 receptive anal sex was estimated by meta-analysis at 1.4% (an average of one
318 transmission event occurred for every 71 exposures), which is at least 10 times higher
319 than for unprotected vaginal intercourse [reviewed [30]]. On another note, the
320 distribution of IgG and IgA varies considerably between different body compartments
321 [31]. While serum contains 3.5 – 14 times more IgG than IgA, IgAs are more prevalent
322 in gastrointestinal tract secretions. Within the intestine, secretions of the digestive part
323 contain more IgA1 than IgA2, whereas secretions of the colon generally possess slightly
324 more IgA2 than IgA1 [31]. In the current study, IgG1 was administered i.v. and thus
325 was distributed systemically as well as into some mucosal fluids. After topical
326 administration, dIgA2 remained localized at the rectal mucosa, because there is no IgA
327 back-transfer from the intestinal lumen across the epithelial barrier. In contrast, the Fc
328 neonatal receptor (FcRn) can shuttle IgG in both directions; it unloads IgG or IgG-
329 immune complex cargo in a pH-dependent manner [reviewed in [32]]. Thus, our
330 passive immunization study reflected the compartmentalization of Ab responses and
331 addressed the vulnerability of the rectal mucosa for HIV-1 infection.

332 While a negative correlation was established between circulating anti-HIV-1 Env
333 IgA in the RV144 trial, our data strongly suggest that a successful HIV-1 vaccine must

generate both mucosal IgA and systemic IgG responses. Such a defensive strategy is best described by the military term “defense-in-depth” – an approach to defend a vital core by pre-planned, well-armed, multiple lines of defense that can provide backup in case the frontline is breached. Defense-in-depth against mucosal HIV-1 transmission can be described as follows. When HIV-1 virions first encounter mucus in the mucosal cavity, they are exposed to secretory IgAs (SIgAs), which may either crosslink the virions, prevent virus transcytosis across the epithelial barrier, or simply neutralize virus. In case some virions remain free, they can penetrate the mucus and the epithelium by transcytosis or other mechanism. Such virions then face ubiquitous IgG and may be neutralized by it. The neutralization capacity of antiviral IgG is limited by its affinity and by the accessibility of HIV-1 Env epitopes. In other words, the tissue and/or plasma concentration of neutralizing IgG must be high enough to neutralize incoming virus as it penetrates the mucosa.

In our current study, the concentration of HGN194 IgG1 used for i.v. administration (1.45 mg/kg) was expected to protect approximately half of the RMs from SHIV challenge based on data from our previous study, where 50% of RMs were protected with 1 mg/kg of HGN194 IgG1 [23]. However, the RMs used for the current study had developed low-level cellular immune responses as a consequence of their earlier experience to live virus; in fact, proliferative responses among $CD4^+$ lymphocytes tended to predominate. Thus, the HGN194 IgG1 dose of 1.45 mg/kg was not protective and all the animals of Group B became infected. When applied mucosally (i.r.) as single mAbs in an earlier study [20], IgG1 protected only 33% and dIgA2 17% of RMs, respectively against the same challenge virus. However in the current experiment, the

combination of i.v. IgG1 plus i.r. dIgA2, in which both mAbs were administered at sub-protective doses, completely prevented virus acquisition. It is reasonable to suggest that in this case, part of the challenge virus was neutralized in the rectal lumen by dIgA2 and to a lesser extent by transudated IgG1; the residual virus, which was not neutralized in the lumen and crossed the epithelium, was met and neutralized by IgG1 in tissues or in the circulation. Thus, our RM model of passive immunization with the combination of topically applied dIgA2 and systemically administered IgG1 followed by mucosal SHIV challenge reflects the outcome of a successful HIV-1 vaccine that induces both systemic IgG and mucosal IgA responses. Our current study provides proof-of-concept for the defense-in-depth strategy against mucosal transmission of HIV-1.

The results of the current study, taken together with our previous findings regarding the protective role of dIgA1 [20], provide a rational explanation of a possible way of preventing HIV-1 acquisition if active vaccination were to generate both mucosal IgA and systemic antibody responses. In this regard, to achieve the complete prevention of HIV-1 infection, a successful vaccine must induce different immune effectors, including HIV-1 Env-specific Abs in mucosal secretions as frontline defense, with back-up provided by neutralizing Abs in tissues and in the circulation and cytotoxic T cells, thus generating defense-in-depth.

Conclusions

Passive immunization with mAb HGN194 IgG1 given systemically together with HGN194 dIgA2 given mucosally completely protected all RMs from high-dose mucosal SHIV challenge, while no monkey given the IgG1 mAb i.v. alone was protected. These results together with our previous findings regarding the protective role of mucosal dIgA1 [20] provide proof-of-concept for defense-in-depth against mucosal transmission of HIV-1.

Materials and Methods

Cell lines, reagents, and virus

The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.. A3R5 cells were kindly provided by Dr. David Montefiori. MAb Fm-6 and VRC01 were kindly provided by Drs. Wayne Marasco (Dana-Farber Cancer Institute) and John Mascola (Vaccine Research Center, NIH), respectively. The SHIV-1157ipEL-p stock (grown in RM PBMC) had a p27 concentration of 792 ng/ml and 7.8 x 10^5 50% tissue culture infectious doses ($TCID_{50}$)/ml as measured in TZM-bl cells. Recombinant HGN194 mAb forms were prepared as described previously [20].

Lymphocyte proliferation assay

PBMC were stained with CFSE (CellTraceTM CFSE Cell Proliferation Kit, Invitrogen) and incubated with or without SIVmac239 Gag peptides (2 μg/ml for each peptide). The peptides (obtained through ARRRP) were 15-mers with an 11-amino acid overlap

between sequential peptides and represented the complete protein sequence. Cells without any stimuli were used to determine background proliferation. After incubation for 5 days at 37^{o}C, cells were stained with anti-CD3-Alexa Fluor 700 (clone SP34-2), anti-CD4-PerCP (clone L200), and anti-CD8-PE (clone RPA-T8) Abs (all from BD Pharmingen). After fixation, at least 10,000 $CD3^{+}$ cells were acquired by flow cytometry, and data were analyzed using FACSDiva (BD Biosciences) software. The percentages of proliferating $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ cells were determined by CFSE dilution; background proliferation (without stimulation) was subtracted.

ELISAs

To evaluate HGN194 IgG1 pharmacokinetics, ELISA plates (Nunc) were coated with 1 µg/ml of SHIV-1157ip gp120 in PBS. After washing, plates were blocked with 4% Non-fat dry milk (Bio-Rad), 0.05% Tween-PBS (blocking buffer). Plates were then incubated with serial dilutions of RM plasma samples in triplicates. HGN194 IgG1 was included as a standard ranging from 0.1 to 31 ng/ml. After washing, plates were developed by incubation for 1 h with goat anti-human IgG HRP-conjugated Ab (Jackson Immunoresearch) or goat anti-human HRP-conjugated Ab that had been adsorbed with monkey IgG (Southern Biotech) to avoid cross-reactivity with monkey anti-gp120 Abs. Color reaction was performed with TMB solution (Life Technologies).

Analysis of RM plasma binding to SHIV-1157ip gp120 was done essentially as described above. Plates were coated with gp120, blocked and incubated with RM plasma samples at different dilutions. To detect binding, plates were incubated with

mouse monoclonal anti-monkey IgG HRP-conjugated Ab with no cross-reactivity to human IgG (Southern Biotech) and developed with TMB solution.

To evaluate RM anti-human IgG responses, plates were coated with 1 µg/ml of HGN194 IgG1 in carbonate buffer, pH 9.6. After blocking and incubation with RM plasma samples, plates were probed mouse monoclonal anti-monkey IgG HRP-conjugated Ab with no cross-reactivity to human IgG (Southern Biotech) and developed with TMB solution.

Animals

RMs were housed at the Yerkes National Primate Research Center (YNPRC, Atlanta, GA) in accordance with standards of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal experiments were approved by the Institutional Animal Care and Use Committees at Emory University and the Dana-Farber Cancer Institute (DFCI) via a Collaborating Institution Animal Use Agreement. Blood was collected under ketamine or Telazol anesthesia.

Passive immunization and mucosal SHIV-1157ipEL-p challenge

All RMs were Mamu B*008 and B*017 negative and aged between 12 to 16 months at the time of challenge. Mamu A*001-positive animals were evenly distributed in each group, as were RMs with different FcγRIIIa genotypes (Table 1). As depicted on Figure 3, Group A RMs (n=6) were treated i.v. with 1.45 mg/kg of HGN194 IgG1 at –24 h, and i.r. with 1.25 mg (in 2.1 ml of PBS) of HGN194 dIgA2 30 min before challenge. The six macaques of Group B were treated i.v. with 1.45 mg/kg of HGN194 IgG1 only at –24 h.

448 The control Group C consisted of two untreated animals. All monkeys were challenged
449 i.r. with 31.5 50% AID_{50} of SHIV-1157ipEL-p [24].
450
451 **Plasma viral RNA levels**
452 Plasma viral RNA levels were measured as described [25, 33].
453
454 **In vitro neutralization assays**
455 For all the assays, mAbs were incubated with virus for 1 h at 37°C and then the cells
456 were added to the mixture. The TZM-bl assay was performed as described [34]. In
457 brief, virus was added to cells in the presence of DEAE-dextran (Sigma), washed 1x on
458 day 1 and luminescence was measured on day 2 using luciferase substrate Bright-Glo
459 (Promega). The A3R5 cell-based assay was performed as described [35] with
460 NL.LucR-1157ipEL virus encoding the *env* gene of SHIV-1157ip-EL envelope [36] and
461 *Renilla* luciferase [37]. Human PBMC-based assays were performed as described [23].
462
463 **Inhibition of transcytosis**
464 HEC-1A cell (ATCC) monolayers were created on 0.4 μm polyethylene terephthalate
465 (PET) membrane hanging transwell inserts (Millipore). Electrical resistance of >400
466 mOhms/cm^2 across the membrane confirmed monolayer integrity. Cell-free SHIV-
467 1157ipEL-p (2 ng/ml of p27) was preincubated for 1 h at 37ºC alone or with various
468 concentrations of HGN194 dIgA1, HGN194 dIgA2, or IgG1, or control IgG1 Fm-6. Next,
469 virus or vius/mAb mixtures were added to the apical surface of the cell monolayer in the

upper chamber. After 12 h, fluid in the lower chamber ("subnatant fluid") was collected and used to measure viral RNA copy numbers by RT-PCR [25, 33].

Statistical Analysis

Statistical analyses were performed using Graph Pad Prism for Windows, version 6 (Graph Pad Software Inc., San Diego, CA).

List of abbreviations

Ab, antibody; mAb, monoclonal antibody; nmAb, neutralizing monoclonal antibody; ADCC, antibody-dependent cellular cytotoxic activity; AID_{50}, 50% animal infectious doses; C1, first conserved region; CTL, cytotoxic T-lymphocyte; dIgA, dimeric IgA; Env, envelope; FcγR, Fcγ receptor; FcRn, Fc neonatal receptor; PBS, phosphate buffered saline; HIV-C, HIV-1 clade C; IC_{50}, 50% inhibitory concentration; IC_{90}, 90% inhibitory concentration; Ig, immunoglobulin; i.r., intrarectal; i.v., intravenous; RM, rhesus monkey; SHIV, simian-human immunodeficiency virus; $TCID_{50}$, 50% tissue culture infectious dose; V1V2, variable loops 1 and 2; vRNA, viral RNA.

Competing interests

A. Lanzavecchia is the scientific founder of Humabs LLC, a company that develops human Abs for treatment of infectious diseases. D. Corti and G. Agatic are currently employees of Humabs. A. Lanzavecchia hold shares in Humabs.

Authors' contributions

Contribution: A.M.S., J.D.W., Q.S., R.A.W., D.C., A.L., J.L.H. and R.M.R conceived the study and designed the experiments; A.M.S., J.D.W., H.K.V., S.K.L., S.T., M.Z., G.H., and B.C.B. performed the experiments; F.V. managed primate experiments; S.G. and D.N.F. performed transcytosis analysis; G.A., D.C. and A.L. prepared mAbs; A.M.S. and R.M.R analyzed the data and wrote the manuscript.

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659 **Table 1 Group reassignment of virus-experienced uninfected RMs**

Groups	Animal	HGN194 version	Type of previous mAb treatment	MHC allele			TRIM5α genotype restriction	Reference
				A*001	B*008	B*017		
Group A	RBk-14	$IgG1_{wt}$	i.v.	-	-	-	moderate	-
IgG1+dIgA2	RIr-14	$IgG1_{kif}$	i.v.	-	-	-	high	-
	RKv-14	$IgG1_{wt}$	i.r.	-	-	-	high	[20]
	RLz-14	dIgA1	i.r.	-	-	-	high	[20]
	RNv-14	dIgA1	i.r.	+	-	-	moderate	[20]
	RWw-14	dIgA1	i.r.	-	-	-	high	[20]
Group B	RAy-14	dIgA1	i.r.	-	-	-	moderate	[20]
IgG1	RCw-14	$IgG1_{wt}$	i.r.	-	-	-	moderate	[20]
	RIk-14	$IgG1_{wt}$	i.v.	+	-	-	moderate	-
	ROw-14	dIgA1	i.r.	-	-	-	high	[20]
	RUq-14	$IgG1_{kif}$	i.v.	-	-	-	high	-
	RYv-14	dIgA2	i.r.	-	-	-	high	[20]
Group C	REo-14	$IgG1_{wt}$	i.v.	+	-	-	moderate	-
controls	RIm-14	$IgG1_{kif}$	i.v.	-	-	-	moderate	-

660 All RMs had no anti-HIV Env Ab responses at the time of the 2^{nd} virus challenge. $IgG1_{wt}$,

661 wild type of HGN194 IgG1; $IgG1_{kif}$, afucosylated version of HGN194 IgG1.

662

Table 2 Concentration and IC_{50} of HGN194 IgG1 in RM plasma on the day of virus challenge

Groups	Animal #	IgG1 concentration, µg/ml	Plasma IC_{50}, µg/ml
Group A	RBk-14	4.0	0.54
IgG1+dIgA2	RIr-14	2.5	ND
	RKv-14	2.8	ND
	RLz-14	2.5	0.32
	RNv-14	3.2	0.86
	RWw-14	4.9	0.30
Group B	RAy-14	3.5	0.53
IgG1	RCw-14	2.4	0.41
	RIk-14	3.9	ND
	ROw-14	3.6	0.56
	RUq-14	3.2	0.40
	RYv-14	2.6	0.32

Plasma IC_{50} concentrations were determined using the concentration of mAb in RM plasma on the day of challenge and the dilution of this plasma sample showing 50% of neutralization in TZM-bl cell assay. Calculations were performed in respect of neutralization obtained with the plasma sample from the same RM taken before the mAb administration at the same dilution. ND, not determined. Experiment performed in triplicate.

Figure legends

Figure 1 Antiviral T-cell responses after previous SHIV-1157ipEL-p challenge [20]. PBMC were stimulated with overlapping peptides representing SIVmac239 Gag and proliferation of $CD4^+$ and $CD8^+$ cells was measured using the CFSE dilution method as described in Materials and Methods. The y-axis indicates % proliferating cells. PBMC isolated from two naïve macaques (RCy-5 and RSf-12) were used as a negative control and PBMC from a previously vaccinated, aviremic animal RAt-9 [38] served as a positive control, respectively. **Panel A**. Positive (RAt-9) [38] and negative (RCy-5 and RSf-12) controls. **Panel B**. T-cell responses of RMs that had received wild-type ($IgG1_{wt}$) or afucosylated ($IgG1_{kif}$) versions of HGN194 IgG1 systemically (i.v.) (unpublished data). **Panel C.** T-cell responses of animals that had previously received HGN194 IgG1, dimeric IgA1 or dimeric IgA2 topically (i.r.) [20].

Figure 2 Antibody responses in RMs previously given passive immunization with different forms of HGN194 [20]. Panels A and B, Only animals that had received HGN194 systemically were analyzed. Mucosally treated RMs had been tested earlier and HGN194 had not been detected in the plasma (data not shown). Red bars, RMs assigned to Group A of the current study; blue bars, RMs assigned to Group B; black bars, RMs assigned to Group C. Solid bars show recipients of HGN194 $IgG1_{wt}$ and striped bars depict recipients of IgG_{kif}. **Panel A.** Residual concentration of HGN194 IgG1 at different time points after administration. HGN194 IgG1 was used as a standard. Secondary goat anti-monkey HRP-conjugated Ab was RM IgG adsorbed. **Panel B.** RM anti-human IgG responses at different time points after HGN194 IgG1 i.v.

696 administration. Grey bars, goat anti-human Ab served as a positive control; +C, positive
697 control (goat anti-human Ab HRP-conjugated). **Panels C and D.** HIV Env binding
698 ELISA analysis of RM plasma samples collected at different time points after virus
699 challenge. **Panel D**. Blue, Group B RM plasma samples. SHIV-1157ip gp120 served
700 as antigen. **Panel C**. Red, Group A RM plasma samples. Open bars, pooled naïve RM
701 plasma was used as a negative control; grey bars, plasma of RRi-11 [39] was used as
702 positive control. The secondary Ab was mouse anti-monkey HRP-conjugated
703 secondary Ab with minimal cross-reactivity to human IgG.
704
705 **Figure 3 Study timeline and design.** Three groups of RMs were enrolled. Group A (n
706 = 6) received the combination of i.v. HGN194 IgG1 (1.45 mg/kg); and i.r. HGN194 dIgA2
707 (1.25 mg). Group B RMs (n = 6) received i.v. HGN194 IgG1 (1.45 mg/kg) only. Group
708 C (n = 2) RMs served as virus-only controls. Black arrow, mAb administrations; yellow
709 arrow, 24 h after IgG1 administration and 30 min after dIgA2 topical application, if any,
710 animals were challenged i.r. with 31.5 AID_{50} of SHIV-1157ipEL-p.
711
712 **Figure 4 The combination of HGN194 IgG1+dIgA2 completely protects RMs from**
713 **high-dose mucosal virus challenge. A.** Red, viral RNA loads for individual RMs for
714 Group A (IgG1+dIgA2); blue, vRNA loads for Group B (IgG1) RMs; black, vRNA loads
715 for Group C (controls) RMs. **B.** Kaplan-Meier analysis of time until vRNA load
716 exceeded 50 copies/ml. Log rank test significance P value is indicated. Red, Group A;
717 blue, Group B.
718

Figure 5 Analysis of HGN194 IgG1 levels in plasma. Panel A. HGN194 IgG1 pharmacokinetics in RM groups. Black arrow indicates SHIV-1157ipEL-p challenge; red, RM of Group A; blue, RMs of Group B. **Panel B.** Analysis of HGN194 IgG1 half-life in RMs. Red, RMs of Group A; blue, RMs of Group B. Statistical analysis was performed by Mann-Whitney test ($P < 0.05$).

Figure 6 The combination of HGN194 IgG1+dIgA2 neutralizes virus similarly to the individual mAbs and does not inhibit virus transcytosis. A-C. Neutralization of SHIV-1157ipEL-p by HGN194 IgG1, dIgA2 and combination of both. The concentration of IgG1+dIgA2 combination is the sum of concentrations of individual mAbs. MAbs VRC01 and Fm-6 were used as positive and negative controls, respectively (not shown). **A.** Human PBMC-based assay; **B.** TZM-bl cell assay; and **C.** A3R5 cell assay. **D.** Inhibition of transcytosis. Solid lines, HGN194 IgG1; dashed lines, isotype control mAb Fm-6. Both IgG1 Abs ranging from 1 to 100 µg/ml were incubated with the virus alone (blue, HGN194; light blue, Fm-6), or together with 200 µg/ml of dIgA2 (red, HGN194; light red, Fm-6), positive control dIgA1 (grey, HGN194; light grey, Fm-6). Next, virus or virus/mAb mixtures were added to the HEC-1A cell monolayer. Twelve h later, vRNA copy numbers were measured. Percent of transcytosis inhibition was calculated in comparison with the number of vRNA copies determined for wells with virus alone. Negative values on Y axis show percent transcytosis enhancement. All experiments were repeated at least twice.

Figure 1

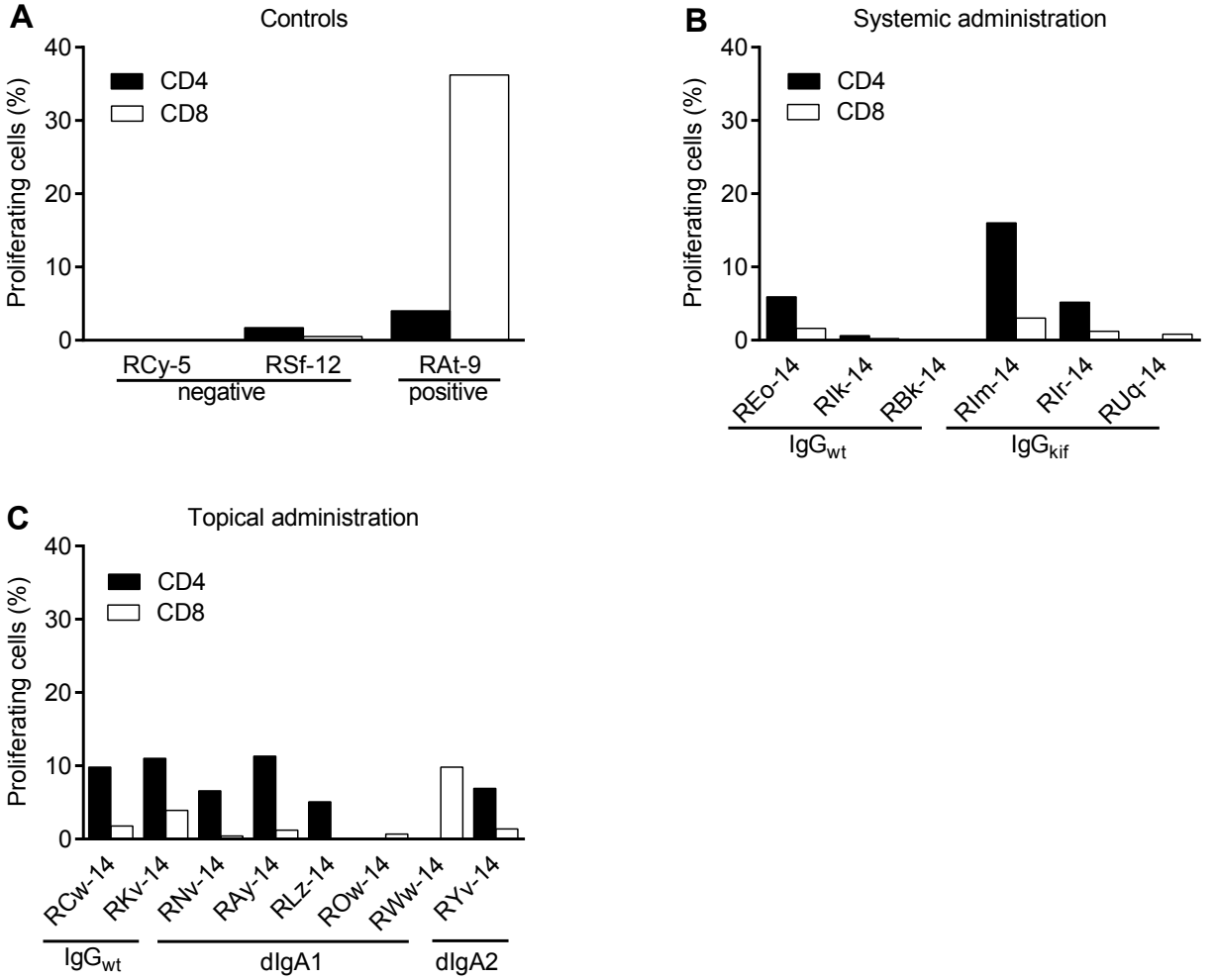

A
Controls
CD4
CD8
Proliferating cells (%)
40
30
20
10
0
RCy-5
RSf-12
negative
RAt-9
positive
B
Systemic administration
CD4
CD8
Proliferating cells (%)
40
30
20
10
0
REo-14
RIk-14
RBk-14
RIm-14
RIr-14
RUq-14
IgGwt
IgGkif
C
Topical administration
CD4
CD8
Proliferating cells (%)
40
30
20
10
0
RCw-14
RKv-14
RNv-14
RAy-14
RLZ-14
ROw-14
RWw-14
RYv-14
IgGwt
dIgA1
dIgA2

Figure 2

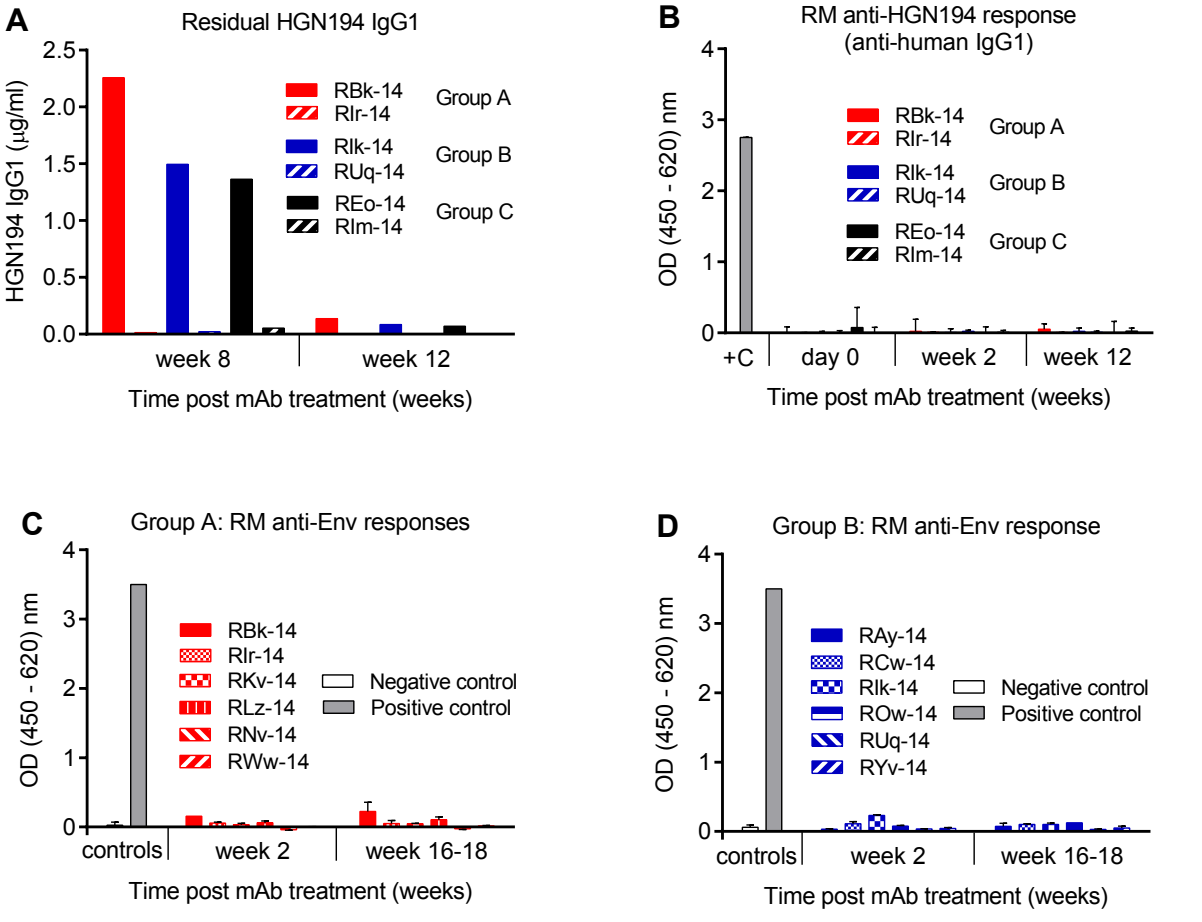

A
Residual HGN194 IgG1
HGN194 IgG1 (µg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
RBk-14
RIr-14
Group A
RIk-14
RUq-14
Group B
REo-14
RIm-14
Group C
week 8
week 12
Time post mAb treatment (weeks)
B
RM anti-HGN194 response
(anti-human IgG1)
OD (450 - 620) nm
4
3
2
1
0
RBk-14
RIr-14
Group A
RIk-14
RUq-14
Group B
REo-14
RIm-14
Group C
+C
day 0
week 2
week 12
Time post mAb treatment (weeks)
C
Group A: RM anti-Env responses
OD (450 - 620) nm
4
3
2
1
0
RBk-14
RIr-14
RKv-14
RLz-14
RNv-14
RWw-14
Negative control
Positive control
controls
week 2
week 16-18
Time post mAb treatment (weeks)
D
Group B: RM anti-Env response
OD (450 - 620) nm
4
3
2
1
0
RAy-14
RCw-14
RIk-14
ROw-14
RUq-14
RYv-14
Negative control
Positive control
controls
week 2
week 16-18
Time post mAb treatment (weeks)

Figure 3

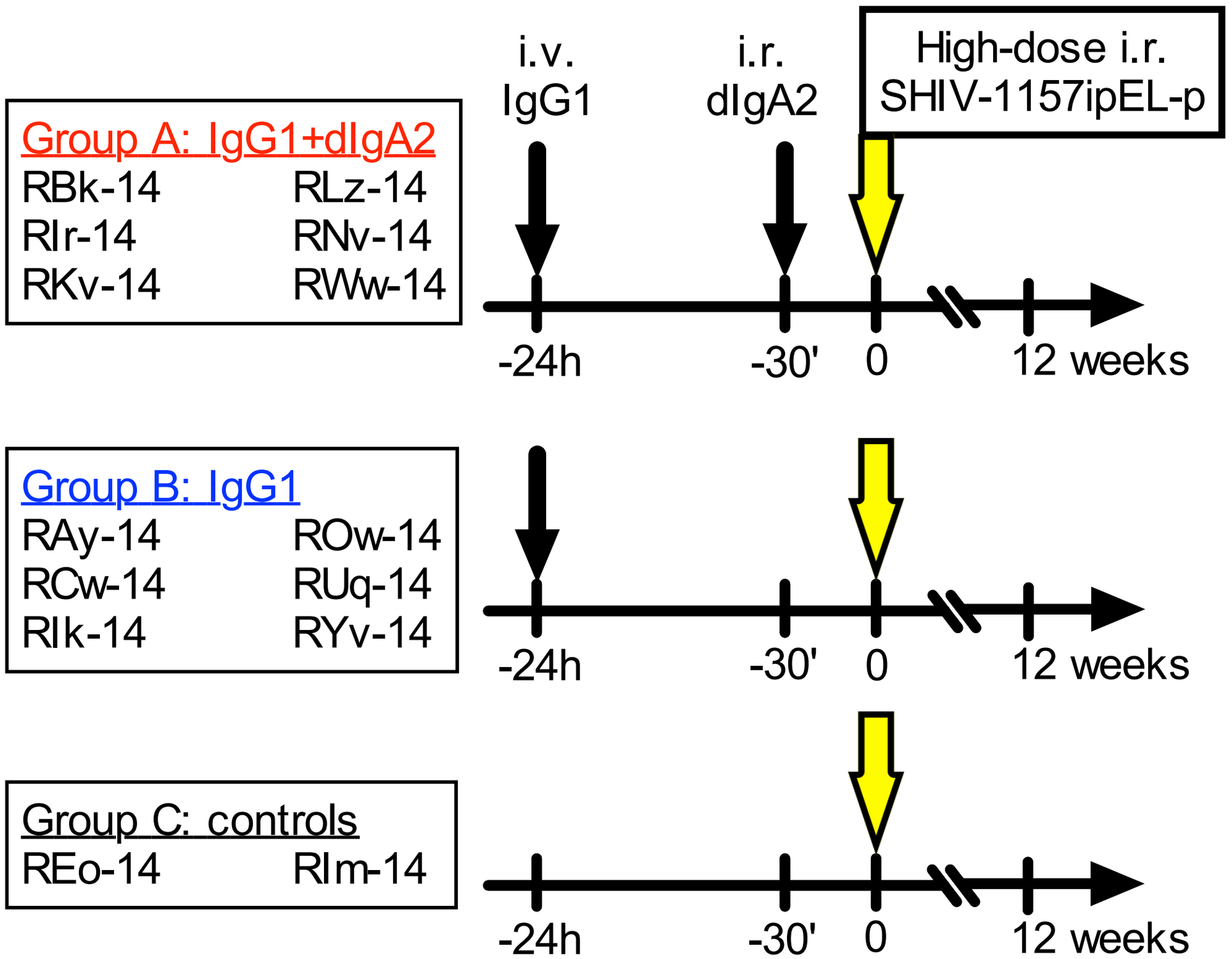

Group A: IgG1+dIgA2
RBk-14
RLz-14
RIr-14
RNv-14
RKv-14
RWw-14
i.v.
IgG1
i.r.
dIgA2
High-dose i.r.
SHIV-1157ipEL-p
-24h
-30'
0
12 weeks
Group B: IgG1
RAy-14
ROw-14
RCw-14
RUq-14
RIk-14
RYv-14
-24h
-30'
0
12 weeks
Group C: controls
REo-14
RIm-14
-24h
-30'
0
12 weeks

Figure 4

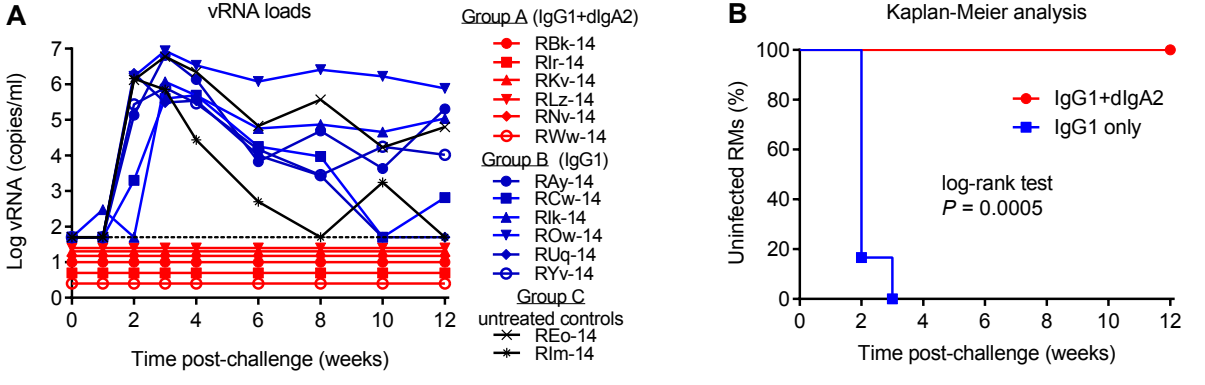
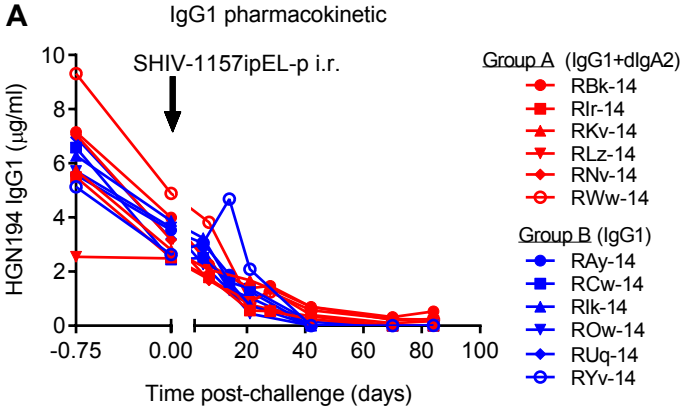

A
vRNA loads
Log vRNA (copies/ml)
Time post-challenge (weeks)
Group A (IgG1+dIgA2)
RBk-14
RIr-14
RKv-14
RLz-14
RNv-14
RWw-14
Group B (IgG1)
RAy-14
RCw-14
RIk-14
ROw-14
RUq-14
RYv-14
Group C
untreated controls
REo-14
RIm-14
B
Kaplan-Meier analysis
Uninfected RMs (%)
IgG1+dIgA2
IgG1 only
log-rank test
P = 0.0005
Time post-challenge (weeks)

Figure 5


A
IgG1 pharmacokinetic
SHIV-1157ipEL-p i.r.
HGN194 IgG1 (μg/ml)
Time post-challenge (days)
Group A (IgG1+dIgA2)
RBk-14
RIr-14
RKv-14
RLz-14
RNv-14
RWw-14
Group B (IgG1)
RAy-14
RCw-14
RIk-14
ROw-14
RUq-14
RYv-14

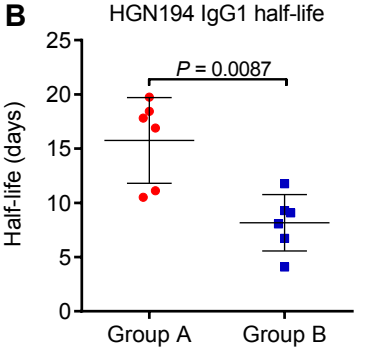
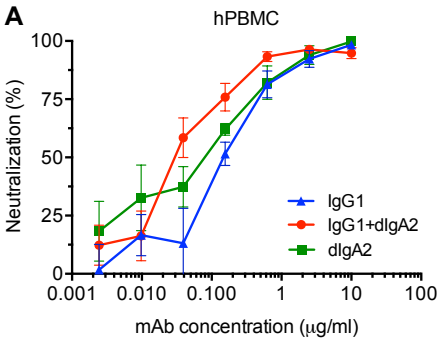
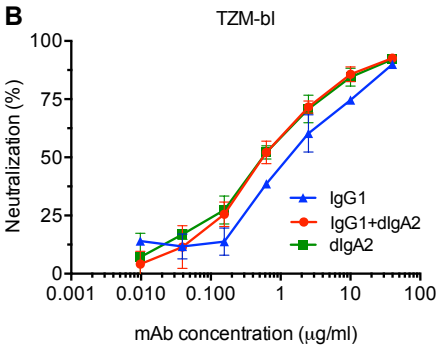
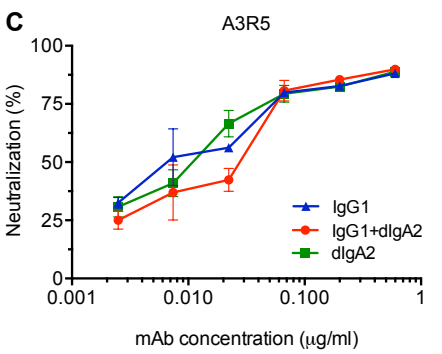
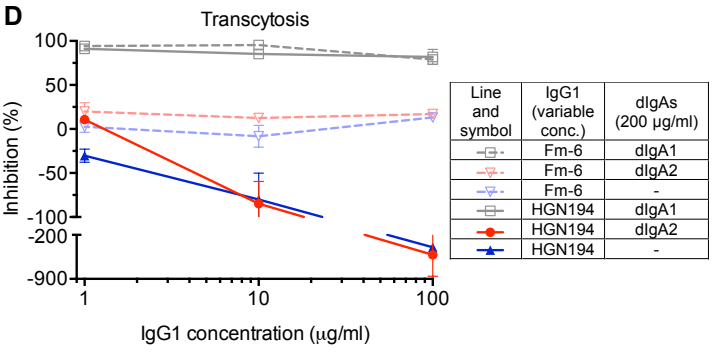

B
HGN194 IgG1 half-life
P = 0.0087
Half-life (days)
Group A
Group B

Figure 6


A
hPBMC
Neutralization (%)
100
75
50
25
0
0.001
0.010
0.100
1
10
100
mAb concentration (μg/ml)
IgG1
IgG1+dIgA2
dIgA2


B
TZM-bl
Neutralization (%)
100
75
50
25
0
0.001
0.010
0.100
1
10
100
mAb concentration (μg/ml)
IgG1
IgG1+dIgA2
dIgA2


C
A3R5
Neutralization (%)
100
75
50
25
0
0.001
0.010
0.100
1
mAb concentration (μg/ml)
IgG1
IgG1+dIgA2
dIgA2


D
Transcytosis
Inhibition (%)
100
50
0
-50
-100
-200
-900
1
10
100
IgG1 concentration (μg/ml)
Line and symbol
IgG1 (variable conc.)
dIgAs (200 μg/ml)
Fm-6
dIgA1
Fm-6
dIgA2
Fm-6
-
HGN194
dIgA1
HGN194
dIgA2
HGN194
-